International Journal of Food Microbiology 293 (2019) 34–43

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Alternaria species associated to wheat black point identified through a multilocus sequence approach

Stefania Somma[1], Maria Teresa Amatulli[1], Mario Masiello, Antonio Moretti*, Antonio Francesco Logrieco

Institute of Sciences of Food Production (ISPA-CNR), via Amendola 122/O, 70126 Bari, Italy

ARTICLE INFO

Keywords:
Alternaria taxonomy
Alternaria section
Infectoriae section

ABSTRACT

Black point is one of the most important wheat disease and its incidence is increasing worldwide due to climate change too. Among the fungal genera that can cause black point, *Alternaria* is one of the predominant genus, often associated with mycotoxin contamination. The correct identification is the baseline for prevention and control of the disease. Taxonomy of the genus *Alternaria* is not completely clear yet, since its species can be differentiated for few morphological traits and, in some cases, also molecular phylogeny is not very effective in establishing species boundaries. In this study, one-hundred sixty-four strains, isolated from wheat kernels affected by black point sampled worldwide, were analyzed in order to assess their identity. Sequences of elongation factor, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase and allergen alt-a1 genes were used to identify the variability of this population and their phylogenetic relationships. Isolates were grouped in two main clades: the *Alternaria* section, including *A. alternata*, *A. tenuissima* and *A. arborescens* species, and the *Infectoriae* section, that includes the two species *A. infectoria* and *A. triticina*. Comparison of isolates according with their area of isolation did not show a correlation between phylogeny and geographic origin. Indeed, the isolates grouped on the base of only their phylogenetic relationship. Due to the data arisen by our study, we strongly recommend a multilocus sequence approach to define *Alternaria* species, based on common genes and procedures to be unanimously shared by scientific community dealing with *Alternaria* genus. Moreover, we suggest that *A. alternata*, *A. tenuissima*, *A. turkisafria* and *A. limoniasperae* species would be merged in the defined species *A. alternata*. Finally we recommend to consider a taxonomic re-evaluation of the *Infectoriae* section that, for the morphology, sexuality, genetic and mycotoxin profile of the species included, could be defined as different fungal genus from *Alternaria*.

1. Introduction

Alternaria species are fungi distributed worldwide as saprophytes, endophytes and plant pathogens in soil, atmosphere, plant materials and food commodities due to their ability to adapt and survive in environmental conditions also far from their optimals (Aust et al., 1980). As plant pathogens, *Alternaria* spp. have been reported on important crops, including cereals, oil crops, ornamentals, vegetables and fruits (Logrieco et al., 2009). *Alternaria* spp. are also known for the production of a great number of secondary metabolites such as host specific toxins (HST) required for pathogenicity, a wide number of mycotoxins, and several allergens (Thomma, 2003). The most important mycotoxins produced by *Alternaria* are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TA), altertoxins I, II and III (ATX, Logrieco et al., 2009), AAL-toxin (Wang et al., 1996) and stemphyltoxin III (Davis et al., 1991). Toxicity, mutagenicity and genotoxicity of several of these metabolites have been proved both in vitro and in vivo assays (Lehmann et al., 2006; Ostry, 2008; Schrader et al., 2001; Zhou and Qiang, 2008). In particular, AOH and AME are genotoxic, mutagenic, and could cause double DNA strand breaks and cell cycle arrest (citaz.). Moreover, AOH was reported to have immune-modulating effects (Solhaug et al., 2015). Tenuazoic acid can exert cytotoxic, phytotoxic, antitumoral, antiviral, antibiotic and antibacterial effects (Asam et al., 2013) and causes a human hematologic disorder called “onalay”, reported in central and southern Africa (da Cruz Cabral et al., 2016). Moreover, Yekeler et al. (2001) showed that TA could induce precancerous changes in the esophageal mucosa of mice. The sphinganine-analog AAL-toxin has phytotoxic effects, causing

* Corresponding author.
E-mail address: antonio.moretti@ispa.cnr.it (A. Moretti).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijfoodmicro.2019.01.001
Received 21 September 2018; Received in revised form 28 December 2018; Accepted 2 January 2019
Available online 03 January 2019
0168-1605/

apoptosis of mammalian cells interfering with ceramide biosynthesis (Wang et al., 1996). Altertoxin I was acutely toxic in mice and mutagenic to mammalian cell lines (Schrader et al., 2006), while both ATX-I and ATX-III were reported as potent mutagens and tumor promoters (Ostry, 2008). On the other hand, ATX II and STTX III were proved to be mutagens (Fleck et al., 2016), and, finally, *Alternaria* mycotoxins have been associated to colon-rectal cancer, occurring significantly in food consummated by humans affected by this pathology (Huybrechts et al., 2018).

The correct identification of fungal taxa is the baseline for further actions to prevent and minimize the diseases and mycotoxin contamination. The taxonomy of the *Alternaria* genus is a very controversial issue. About 280 species in *Alternaria* genus were described by Simmons (1967, 1992, 1999, 2007), based exclusively on morphological traits of colonies growing in particular on sporulation patterns. Indeed, morphological traits have been for a long time the unique tool to classify strains of *Alternaria* and related genera. This approach is however not always effective, due to the high influence of growth conditions on the morphological features, the high level of similarity among some species and the presence of several strains with intermediate traits (Andrew et al., 2009; Misaghi et al., 1978). Moreover, even species belonging to other fungal genera phylogenetically closed to *Alternaria* were described with the same morphological criteria (Simmons, 1967, 1989, 1990a). As a consequence, many studies have been devoted to *Alternaria* and related genera, often confirming a confused taxonomic back-ground, that has caused a continuous process of taxonomic revision.

Different molecular tools were used to support morphology for the *Alternaria* taxonomy, including DNA fingerprinting techniques (RAPD, PCR-RFLP, AFLP and ISSR) and sequence analysis of rDNA and protein-coding genes, such as glyceraldehyde-3-phosphate dehydrogenase (*gpd*), endopolygalacturonase (*endoPG*), b-tubulin (*tub*), and allergen alt-a1 (*alt-a1*). However, although this wide and differentiated range of approaches, the data obtained were not always congruent (Andersen et al., 2009; Andrew et al., 2009; Hong et al., 2005; Hong et al., 2006; Park et al., 2008; Peever et al., 2004; Somma et al., 2011).

More recently, several authors increased the molecular investigations on wide sets of strains belonging to *Alternaria* and closely related genera, through phylogenetic analyses by using multiple gene approach. Lawrence et al. (2012) investigated the phylogenetic relationships of *Embellisia* and *Nimbya* species with species belonging to *Alternaria*, *Ulocladium*, *Undifilum*, *Crivellia* and *Stemphylium* genera, by analyzing three genes: internal transcribed spacer (*its*), *gpd* and *alt a1*. This study revealed polyphyly within each genus and incongruence with the morphological classification. Moreover, *Alternaria* species were resolved in species-groups. When deeper investigations were carried out within the *Alternaria* genus by using 10 different loci (Lawrence et al., 2013), among which only 5 (*alt-a1*, *gpd*, actin, calmodulin - *caM* - and plasma membrane ATPase), were considered informative for *Alternaria* phylogeny, the previously defined species-groups in *Alternaria* were elevated to the taxonomic status of sections. Eight sections in *Alternaria* genus were defined, *Porri*, *Alternaria*, *Althernaterae*, *Sonchi*, *Gypsophilae*, *Radicina*, *Panax* and *Brassicicola*, paraphyletic with the *Ulocladium* genus, and also other different polyphyletic groups, including *A. infectoriae* species-group, were described (Lawrence et al., 2013). A further investigation on *Alternaria* analyzed phylogenetically strains belonging to 25 different species included in *A. infectoriae* species-group, highly morphologically variable and provided of sexual stage (teleomorphs) (Lawrence et al., 2014). The study grouped all 25 species in a well supported clade, *A. infectoriae* species-group, that included also strains identified as *Pseudoalternaria*, *Chalatospora*, *Embellisia* and *Nimbya*. Interestingly, this clade resulted phylogenetically distant from the clade that grouped other species of *Alternaria*, all lacking the sexual stage. On the other hand, Woudenberg et al. (2013, 2015) defined in *Alternaria* genus 26 sections, among which the *Alternaria* section is characterized by the highest number of morpho-species, and *A. infectoriae* species-group was elevated at Section rank, *Infectoriae* section. The deeper study focused on *Alternaria* section (Woudenberg et al., 2015) was performed by using 7 gene regions: *alt-a1*, *gpd*, *endoPG*, *ITS*, translation elongation factor (*tef*), RNA polymerase II gene (*RPB2*) and an anonymous gene region OPA10–2. In previous studies, species belonging to section *Alternaria*, that were mostly described founded on morphology or host specificity, were not successfully distinguished by sequencing because of their very high level of similarity. Woudenberg et al. (2015) had same difficulties in distinguishing *Alternaria* morphospecies based on sequences of the used housekeeping genes. Therefore, whole-genome sequencing and transcriptome analyses were also used to integrated phylogenetic study. The data generated allowed the authors to define in *Alternaria* a single species complex, *A. arborescens* species complex (AASC), and 11 phylogenetic species, among which *A. alternata*, that alone synonymised 35 not molecularly distinguished morphospecies.

Contamination of wheat kernels by *Alternaria* species and related toxins has been reported in different countries worldwide (Logrieco et al., 2009). *Alternaria alternata*, ubiquitous pathogen on several foodstuffs, and *A. triticina*, known to be among the causal agents of wheat leaf blight in different geographic areas (Mercado Vergnes et al., 2006), both resulted the predominant species associated with grains, although other species of the *Alternaria* and *Infectoriae* sections have been occasionally isolated (Gargouri-Kammoun et al., 2014; Rathod and Chavan, 2010; Vučković et al., 2012). However, all these reports have been more often limited to specific geographical areas or a low number of samples. Moreover, due to the presence of toxigenic species among those belonging to *Alternaria* section, a correct identification of most occurring species on wheat is not only useful to define their identity, based on the new taxonomic system proposed by Woudenberg et al. (2015), but it is also of key importance to assess the toxicological risk caused by the *Alternaria* species associated to wheat.

The aims of this work were: a) to study the genetic diversity of the main *Alternaria* species associated with wheat black point; b) to evaluate the phylogenetic relationships among isolates sampled in Italy and compare them with isolates sampled worldwide; c) to obtain a clearer picture of species borders among the *Alternaria* species affecting wheat, by using a multilocus sequence approach.

2. Materials and methods

2.1. Fungal strains and growth conditions

One hundred sixty-four representative strains of *Alternaria* spp. associated with wheat black point were selected from a wider population deposited in ITEM collection of Institute of Science of Food Production, Bari, Italy (http://ispa.cnr.it/ITEM/Collection/) based on geographic origins (97 from Italy, 6 from Austria, 2 from England, 7 from Hungary, 7 from Croatia, 7 from Greece, 5 from Turkey, 9 from Kazakhstan, 9 from Russia, 4 from Canada, 7 from USA, 4 from Mexico). In addition, 6 strains of *Stemphylium* genus, closely related to *Alternaria*, were included in the analysis as outgroups. The strains were isolated from both healthy and diseased wheat kernels samples collected mainly in Italy and other different Countries worldwide (Table 1). The strains were all isolated according with the procedure described in Amatulli et al. (2013). As reference, strains E.G.S. 34.016 (*A. alternata* type strain), E.G.S. 34.015 (*A. tenuissima* type strain), E.G.S. 39.128 (*A. arborescens* type strain) and E.G.S. 27.193 (*A. infectoria* type strain) were used (Table 2). Other strains, received from the Mycothèque de l'Université catholique de Louvain, Belgium, BCCM/MUCL Environmental and applied mycology (BCCM/MUCL), such as *A. alternata* (MUCL45332 and MUCL42372), *A. tenuissima* (MUCL42464), *A. arborescens* (MUCL45333) and *A. triticina* (MUCL42465 and MUCL 44259) species, listed in Table 2, were included in the analyses (Table 2).

Table 1
Alternaria strains of ISPA collection (164 strains) isolated from wheat worldwide and selected for phylogenetic analysis. The membership to the phylogenetic clade obtained from the analysis of all the strains and shown in Fig. 1 is reported.

Geographic origin	Number of strains	Strains (ITEM number)	Phylogenetic clade
Italy	97	10472-10473-10480-10486-10491-10493-10502-10503-11659-11665-13632-13633-13634-13637-13646-15876-15877-15879-15880-15881-15882-15885-15892-15894-15895-15897-15899-15900-15901-15902-15903-15905-15906-15907-15909-16139-16140	Section *Alternaria*
		10467-10468-10469-10471-10474-10475-10476-10477-10478-10479-10481-10482-10483-10485-10488-10492-10494-10495-10496-10497-10498-10499-10500-10501-10504-10505-10506-10507-10508-10509-10510-10511-10512-10513-13635-13638-13639-13641-13642-13644-13645-13647-13648-15883-15886-15887-15888-15889-15891-15893-15898-15904-15908-16142-16144	Section *Infectoriae*
		11658-11666-13630	Unknown
		10487[a]-11667	*Stemphylium*
Austria	6	15912-15915-15916-15917-15919	Section *Alternaria*
		15914	Section *Infectoriae*
England	2	13201-13202	Section *Infectoriae*
Hungary	7	11618-11620-11624-11625	Section *Alternaria*
		11619-11621-11623	Section *Infectoriae*
Croatia	7	11589-11592-11593	Section *Alternaria*
		11588-11591-11594-11597	Section *Infectoriae*
Greece	7	11608-11610-11612-11614-11617	Section *Alternaria*
		11609	Section *Infectoriae*
		11615	*Stemphylium*
Turkey	5	11630-11631-11632-11633-11635	Section *Alternaria*
Kazakhstan	9	11598-11599-11600-11601-11604-11605-11607	Section *Alternaria*
		11602-11606	Section *Infectoriae*
Russia	9	11668-11669-11670-11672	Section *Alternaria*
		11673-11674-11675-11676	Section *Infectoriae*
		11671	*Stemphylium*
Canada	4	11642-11643-11646	Section *Alternaria*
		11639	*Stemphylium*
USA	7	11649-11652-11655-11650-11656–11657	Section *Alternaria*
		11651	*Stemphylium*
Mexico	4	11680	Section *Alternaria*
		11681-11682	Section *Infectoriae*
		11678	Unknown

[a] Underlined strains belong to *Stemphylium* genus and have been used in the study as outgroup (6 strains).

2.2. DNA extraction and PCR amplification

Each strain was grown in flasks containing 100 ml of Wickerman's medium (glucose 4%, malt extract 0.3%, yeast extract 0.3% and peptone 0.5%) and incubated at 25 °C under shaking (120 rpm). Two days after inoculation, liquid cultures were filtered on Whatman No. 4 filter paper, washed with distilled water, frozen, and lyophilized.

About 15 mg of powdered lyophilized mycelia were used for DNA extraction by using the Wizard Magnetic DNA Purification System for Food (Promega Corp., Madison, WI) according to the manufacture's protocol. Quantity and quality of DNA were checked at Nanodrop (Thermo-Scientific) and by comparison with standard DNA markers (1 kb DNA Ladder, Fermentas GmbH, St. Leon-Rot, Germany) on 0.8% agarose gel.

To select the most informative sequences for multilocus analysis of all *Alternaria* isolates, the 10 reference strains used in this study, listed in Table 2, were analyzed by amplifying and sequencing 8 of the main housekeeping genes usually used for fungal species identification: *its*, *caM*, translation elongation factor 1 α (*tef*), *tub*, histone 3 (*h3*), *endoPG*, *alt-a1*, *gpd*. As a result of this screening, *alt-a1*, *gpd*, *tub* and *tef* were selected as the most informative genes.

Amplification of these four genes were performed with the following primer combinations: alt-for and alt-rev for *alt-a1* (Hong et al., 2005), gpd1 and gpd2 for *gpd* (Berbee et al., 1999), T1 and Bt2b, for *tub*

Table 2
Alternaria strains sequenced in this work to be used as reference in the phylogenetic analyses. *Alternaria* species, other collection number, origin and bibliographic references are reported for each strain.

Alternaria species	Strains number	Other collection number	Type of organism	Source	Geographical origin	Bibliographic references
A. alternata	E.G.S. 34.016	CBS 916.96 ATCC 66981 FRR 5009 IMI 254138 NBRC 32415 VKM F-4343	Type strain	*Arachis hypogaea*	India	Simmons, 2007
A. tenuissima	E.G.S. 34.015	CBS 918.96 ATCC 96828 FRR 5015 IMI 255532 VKM F-4349	Representative isolate	*Dianthus chinensis*	UK	Simmons, 2007
A. arborescens	E.G.S. 39.128	CBS102605 ATCC 204491 VKM F-4344 AS27-31z	Type strain	*Solanum lycopersicum*	USA	Simmons, 2007
A. infectoria	E.G.S. 27.193	CBS 210.86	Type strain	*Triticum aestivum*	UK	Simmons, 2007
A. triticina	MUCL42465	ITEM 15922	–	*Triticum aestivum*	India	Mercado Vergnes et al., 2006

(Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), while for *tef* amplification, Alt-tef1 (CTTGGAGGGAACCATCTTGA) and Alt-tef2 (CTGGTACAAGGGTTGGGAGA) were designed by aligning the *tef* sequences of *F. verticillioides* (FN552074) on *A. brassicicola* (ATCC 96836) genome (genomeportal.jgi-psf.org).

All amplifications were performed in a final volume of 15 µl, using 15 ng of DNA, 0.45 l of each primer (15 mM), 0.3 µl of dNTPs (10 mM) and 0.075 µl of Hot Master Taq DNA Polymerase (1 U/µl; 5Prime). The thermal cycler parameters were: initial denaturation for 2 min at 95 °C followed by 35 cycles of 94 °C for 30 s, annealing for 30 s, 72 °C for 50 s, and a final extension for 7 min at 72 °C. Annealing temperatures were: 58 °C for *alt-a1*, 57 °C for *gpd*, 55 °C for *tub* and *tef*. PCR products were checked on 1,5% agarose gels and visualized with UV after GelRed (Biotium Inc.) staining to confirm the expected products.

2.3. Sequencing and phylogenetic analysis

Sequence analysis was performed with Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) for both strands of each gene and fragments were purified by filtration through Shepadex G-50 (Sigma) before to be sequenced.

Sequences of each strand were combined, edited and merged into composite sequences with BioNumerics (Applied Maths, Kortrijk, Belgium).

Text sequences were then aligned and analyzed with MEGA7 (Kumar et al., 2016).

Phylogenetic trees of single and combined genes were generated using maximum likelihood and bootstrap analysis (1000 replicates, removing gaps) with MEGA7 (Kumar et al., 2016).

2.4. Reference sequences for Alternaria species

Since the genes portions sequenced for phylogenetic analysis didn't overlap with those available in public databases, the Alternaria Genome Database (AGD) was used to download the target fragments from all the available published genomes (Dang et al., 2015), after using the sequence alignment search BLAST. The 22 strains included in our phylogenetic trees as references are listed in Table 3.

Table 3
List of 20 *Alternaria* species reference with sequenced genomes, from Alternaria Genome Database (Dang et al., 2015), used in this work to compare partial genes sequences for phylogenetic analyses.

Species name	Strain codes
A. alternata	ATCC 66891, EGS 34–016, BMP 0269
A. alternata	ATCC 66982, EGS 34–039, BMP 0270
A. arborescens	ATCC 204491, EGS 39–128, BMP 0308
A. brassicicola	ATCC 96836, EGS 42–002, BMP 1950
A. capsici	ATCC MYA-998, EGS 45–075, BMP 0180
A. carthami	BMP 1963, CBS 635.80
A. citriarbusti	EGS 46–140, BMP 2343, SH-MIL-8 s
A. crassa	BMP 0172, ACR1
A. fragariae	BMP 3062, NAF-8
A. gaisen	EGS 90–0512, BMP 2338
A. limoniasperae	EGS 44–159, BMP 2335
A. longipes	EGS 30–033, BMP 0313
A. mali	BMP 3064, IFO8984
A. macrospora	BMP 1949, CH3
A. solani	BMP 0185
A. tagetica	EGS 44–044, BMP 0179
A. tangelonis	EGS 45–080, BMP 2327, BC2-RLR-1 s
A. tenuissima	ATCC 96828, EGS 34–015, BMP 0304
A. tomatophila	BMP 2032, CBS 109156
A. turkisafria	BMP 3436, SH-MIL-20s

ATCC = American Type Culture Collection; EGS = E. G. Simmons.

3. Results

3.1. Analysis of gene sequences

All the strains yielded amplification products of the expected size for all the analyzed genes, about 500 bp for *alt-a1* and about 600 for *gpd*, *tef* and *tub*.

A multiple sequence alignment was performed for each gene, to analyze the sequence variability among the population. The sequences of each gene were cut at the ends to analyze a common fragment for all the strains; however several insertion and deletion fragments (indel) were shown among groups of strains in each gene sequence.

After the multiple alignment of *alt-a1* sequences, ranging from 409 to 469 nucleotides (nt), 473 total sites were obtained. Two different indel portions were observed: one fragment of 6 nt, absent in the strains identified as *A. triticina*, and a bigger portion of 62 nt, absent in the strains identified as *Stemphylium* and used as outgroup.

Sequences of *gpd*, ranging from 542 and 545 nt, gave 570 total sites. Two small indels were observed, one of 3 nt absent in *A. triticina* and *A. infectoria* strains, and one of 2 nt, belonging to *Stemphylium* strains.

From the analysis of *tub* sequences, ranging from 479 to 492 nt, 518 sites were obtained, with 3 evident indels: two indels of 5 and 10 nt, were absent in *A. triticina* and *A. infectoria* strains, and one indel of 4 nt was present only in *Stemphylium* strains.

Only *tef* sequence alignment did not show indels and gave 524 final sites.

To generate the phylogenetic tree from the analysis of all the four genes, the *alt-a1*, *gpd*, *tef* and *tub* gene sequences were concatenated into a super-gene alignment, that gave 2085 total sites.

Data obtained from sequences alignment were also analyzed to evaluate the capability of each gene to be informative. Data of the constant, variable, parsimony-informative and singleton sites, as well as the polymorphic sites, expressed as percentage of variable on total sites, were reported in Table 4. Among the four genes, *alt-a1* resulted the most informative, with 46% of polymorphic sites, followed by *gpd* (34%) and *tub* (33%); only 12% of polymorphic sites were registered for *tef*.

3.2. Phylogenetic analysis

One maximum likelihood phylogenetic tree was generated for each sequenced gene, *alt1a*, *gpd*, *tef* and *tub* (data not showed), and for the concatenated sequences of the 4 genes (Fig. 1).

The phylogenetic tree showed two well defined clades: section *Alternaria* clade, including *A. alternata/A. tenuissima/A. arborescens* reference strains, section *Infectoriae* clade, including *A. infectoria/A. triticina* reference strains. A third clade was constituted by reference species of section *Porri*, while *A. brassicicola* clustered alone. Four strains, ITEM 11658, ITEM 11666, ITEM 11678 and ITEM 13630, more closely related to section *Infectoriae* clade, showed a very high variability. These strains were not solved in a clade and were not identified for lack of reference strains sharing phylogenetic similarity with them. A separate clade included the outgroup strains morphologically identified as *Stemphylium* and one reference strain of *Stemphylium versicarium*. This latter clade was highly variable and, although showed to be more related to *Alternaria* section clade, was phylogenetically located between *Alternaria* and *Infectoriae* sections clades (Fig. 1). Nevertheless, BLAST analysis against NCBI database of the sequences of these strains found 99–100% identity with strains of the genus *Stemphylium/Pleospora*.

Two different further phylogenetic analyses with maximum likelihood method were carried out for 80 strains belonging to *Alternaria* section clade, and for 74 strains belonging to *Infectoriae* section clade, including *Alternaria* species references and one strain of *Stemphylium versicarium* as outgroup in both the analyses.

The phylogenetic tree of *Alternaria* section clade, including *A.*

Table 4
Analysis of sequence data after multiple alignment of allergen alt a1 (*alt-a1*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*), translation elongation factor 1 α (*tef*) and β-tubulin (*tub*) genes, sequenced on 164 *Alternaria* strains isolated from wheat, 5 strains from MUCL collection, and 22 species reference. The analysis was carried out with MEGA7 software.

Gene sequence	Total sites	Constant sites	Variable sites	Parsimony-informative sites	Singleton sites	Polymorphic sites (%)
alt-a1	473	251	220	183	37	46
gpd	570	374	192	152	40	34
tef	524	461	63	44	19	12
tub	518	335	172	127	45	33
tot	2085	1421	647	506	141	31

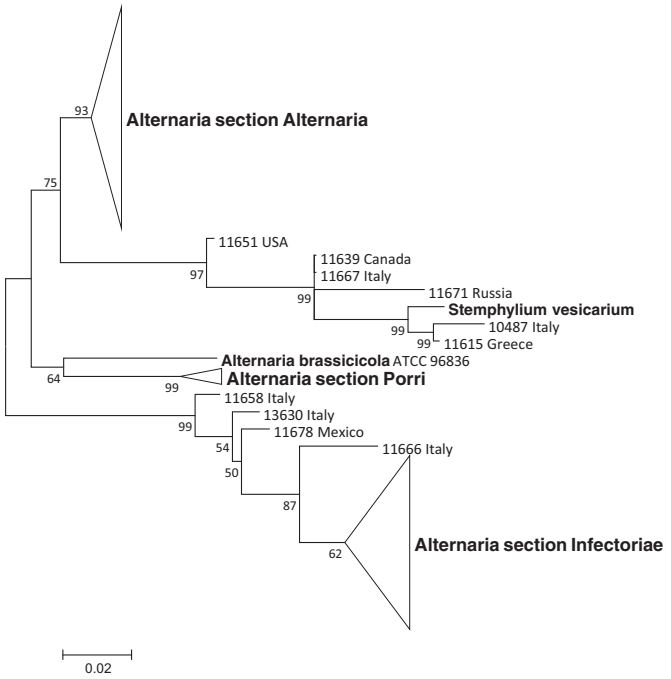

Fig. 1. Schematic phylogenetic tree obtained by maximum likelihood analysis (bootstrap 1000 replicates) of combined *alt-a1*, *gpd*, *tef* and *tub* gene sequences of 164 unknown strains, 5 strains from MUCL collection, and 22 species reference. Most of ITEM strains are included in 2 main clades: 80 strains in *Alternaria* section and 74 strains in *Infectoriae* section. There were a total of 2085 positions in the final dataset. The percentage of trees in which the associated taxa clustered together is shown next to the branches (cut off value at 50). Geographical origin of strain is reported near the ITEM number.

alternata/A. tenuissima/A. arborescens strains, is reported in Fig. 2. The analysis of the four combined sequences involved 108 total strains and generated 2083 total nucleotide positions in the final dataset. The sequences of the reference strains for *A. alternata*, *A. tenuissima* and *A. arborescens* species, sequenced in this work (Table 2), and sequences of same species, downloaded from the AGD (Table 3) and referred to the same strains (i.e. *A. alternata* EGS 34.016 = bmp 0269 = ATCC 66981), matched perfectly. Thus in the phylogenetic tree only one sequence for same strain was reported.

The 80 *Alternaria* strains were resolved in 4 different groups, named Group 1, Group 2, Group 3, Group 4 in Fig. 2. In particular, most of strains (53 ITEM strains) shared a very high similarity and grouped together in Group 1, in which reference strains for *A. alternata* (E.G.S. 34.016) and *A. tenuissima* (E.G.S. 34.015), besides MUCL 42372 and 45332 (*A. alternata*) and MUCL 42464 (*A. tenuissima*) strains, were included. The very high level of homology of these strains did not allow to distinguish between *A. alternata* and *A. tenuissima* species. Moreover, in the same group clustered also the species *A. turkisafria*, and *A. limoniasperae*.

The ex-type strain of *A. arborescens* (E.G.S. 39.128) grouped together with 3 strains (ITEM 11668, ITEM 15894, ITEM 15907) in Group 2.

In Group 3, supported by 99 of bootstrap value, 17 strains clustered with the reference species *A. citriarbusti*, *A. mali*, *A. tangelonis*, *A. longipes*, *A. gaisen* and *A. fragariae*, and with MUCL 45333 (received as *A. arborescens*) and MUCL 44259 (received as *A. triticina* by the MUCL Collection, but reported as *A. arborescens* in the reference paper cited in the collection, Mercado Vergnes et al., 2006). Moreover, the strains of Group 3 shared the highest similarity with *A. citriarbusti* and *A. mali* species reference strains.

A very strongly supported group (100 bootstrap value), Group 4, included 7 strains, which were not identified for the lack of reference strains in AGD.

Finally, a separate clade, well supported (100 of bootstrap value), was formed by the reference strains of the species belonging to the section *Porri*, namely, *A. tagetica*, *A. capsici*, *A. crassa*, *A. macrospora*, *A. solani*, *A. carthami* and *A. tomatophila*.

The species *A. brassicicola* was clearly separated from all the other species included in the analysis.

The analysis of *Infectoriae* section clade obtained from the first pylogenetic analysis on all the *Alternaria* population strains generated 2050 total nucleotide positions in the final dataset, involving 97 total sequences, which included 74 ITEM strains, the *Alternaria* species references and *Stemphylium* as outgroup (Fig. 1).

The tree generated by the phylogenetic analysis of *Infectoriae* section clade is shown in Fig. 3. These strains were separated in 2 different groups, named Group 5 and Group 6. A big clade, with a bootstrap value of 85, includes 62 strains: 2 strains (ITEM10498 and ITEM 13641) clustered together, and the other 60 strains formed 5 different clusters, though not well supported. One of these clusters, made by 7 strains all from Italy (ITEM 10467, ITEM 10482, ITEM 10485, ITEM 10488, ITEM 10499, ITEM 13638 and ITEM 15883), included the reference strain of *A. triticina* MUCL 42465. A different cluster, named Group 6 in Fig. 3, was formed with 10 strains very closed to *A. infectoria* E.G.S. 27.193, while, 2 strains clustered alone: ITEM 11676, located between Group 5 and Group 6, and ITEM 13645, highly different from all the other strains reported in this phylogenetic tree.

Strains of *Infectoriae* section clade (Fig. 3) shared a lower level of similarity among them if compared to those of *Alternaria* section clade (Fig. 2). On the contrary, the clustering is not well supported. Both the phylogenetic trees of *Alternaria* and *Infectoriae* sections were rooted on the *Stemphylium* reference strain used as outgroup.

Finally, no correlation between geographic origin of the strains analyzed in this study and the clades generated was found (Figs. 2 and 3).

4. Discussion

Reports on the natural occurrence of *Alternaria* species and related mycotoxins on wheat are increasing worldwide. Among the species identified, *A. alternata* has been reported as the most spread causal agent, being associated to the so-called black point disease of wheat kernels (Bensassi et al., 2009; Gannibal et al., 2007; Li and Yoshizawa, 2000; Mašková et al., 2012; Patriarca et al., 2007; Scott et al., 2012;

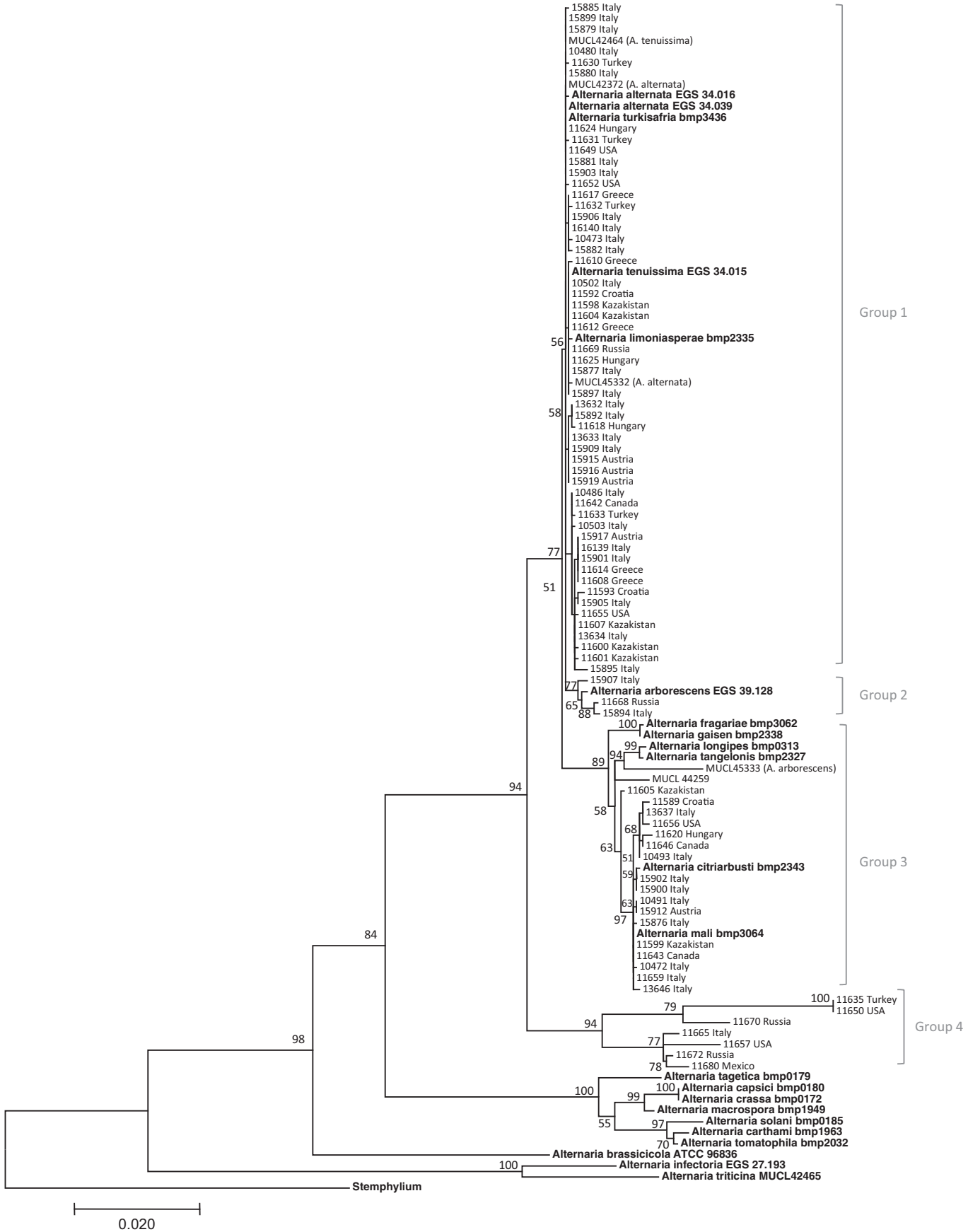

Fig. 2. Phylogenetic tree generated by maximum likelihood analysis (bootstrap 1000 replicates) of combined *alt-a1*, *gpd*, *tef* and *tub* gene sequences of 80 strains of the *Alternaria* section clade, 5 strains from MUCL collection, and 22 species reference. There were a total of 2083 positions in the final dataset. The percentage of trees in which the associated taxa clustered together is shown next to the branches (cut off value at 50). Geographical origin of strain is reported near the ITEM number.

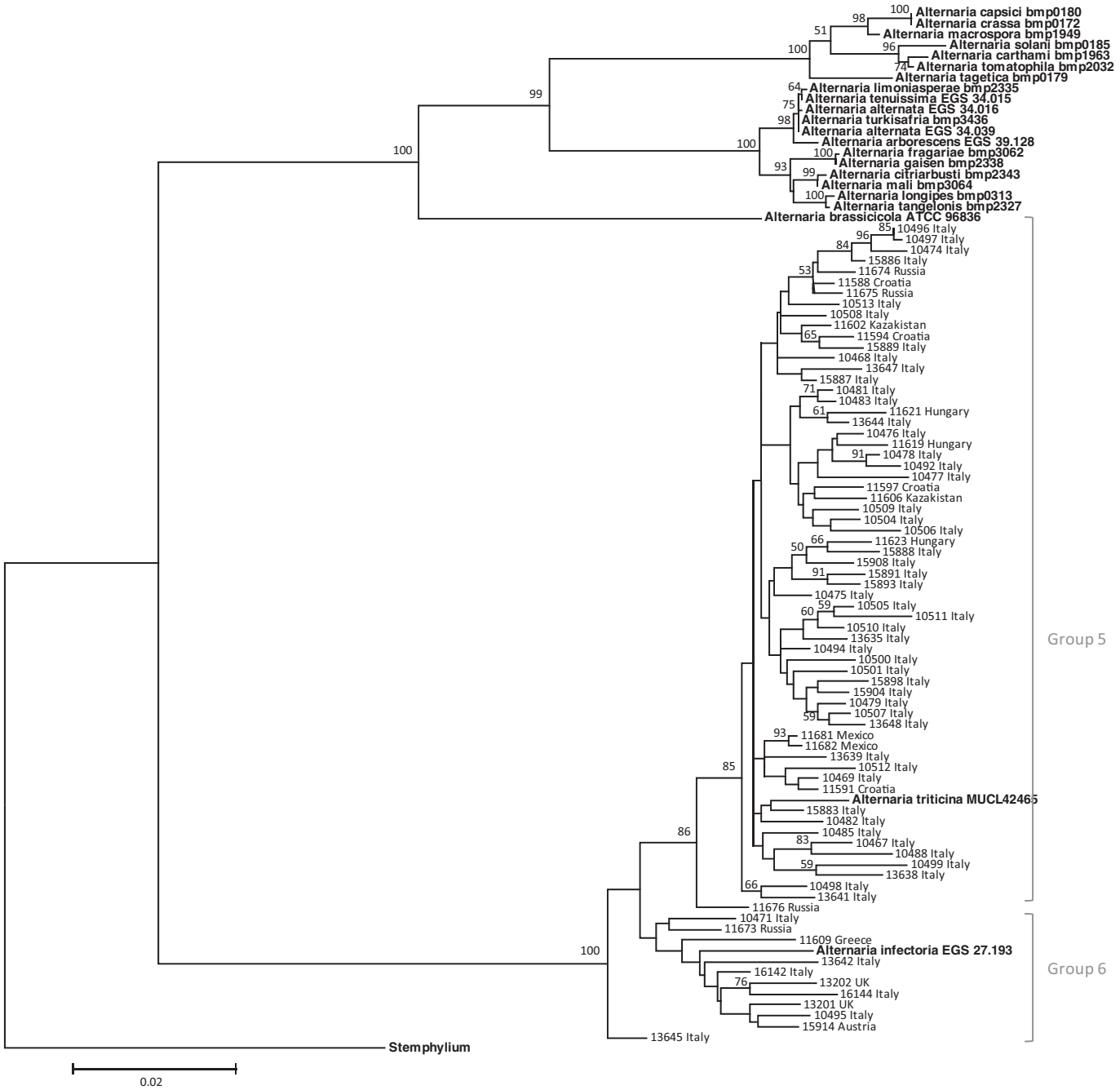

Fig. 3. Phylogenetic tree generated by maximum likelihood analysis (bootstrap 1000 replicates) of combined *alt-a1*, *gpd*, *tef* and *tub* gene sequences of 74 strains of the *Infectoriae* section clade and 22 species reference. There were a total of 2091 positions in the final dataset. The percentage of trees in which the associated taxa clustered together is shown next to the branches (cut off value at 50). Geographical origin of strain is reported near the ITEM number.

Tralamazza et al., 2018; Vučković et al., 2012).

On the contrary, *A. triticina*, firstly reported in India (Prasada and Prabhu, 1962), is still considered mostly a severe foliar disease agent of wheat in India (Sharma et al., 1998; Shukla et al., 2006) and China (Guo, 2005). However, since there is no consistent evidence of *A. triticina* pathogenicity on wheat kernels and it produces only phytotoxic compounds and no mycotoxins, *A. triticina* is generally considered as a weak pathogen of wheat (Wiese, 1987; CPC, 2018).

Alternaria species grow under a wide range environmental conditions, also far from their optimal growth parameters (Aust et al., 1980; Rotem et al., 1978). Therefore, these species can adapt their ecology and compete in the colonization of new habitats, even when exposed to the climate changes that are affecting the ecosystem equilibrium in the recent years. Furthermore, *Alternaria* occurrence on wheat generates great concern in scientific community for both the ability to contaminate a huge number of natural and processed products and to produce mycotoxins (Amatulli et al., 2013). However, although evidence on toxicity of *Alternaria* mycotoxins animals and humans has been collected through years (Liu et al., 1991), specific regulations regarding *Alternaria* toxins have not been established yet at worldwide level. In 2011, EFSA published a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food (EFSA, 2011), but an urgent need of regulation was not acknowledged, although the study suggested to keep a periodic monitoring of the situation. In particular, great attention should be dedicated to crops which are the basis of human diet, such as wheat.

In the present work, we analyzed a wide population of *Alternaria* strains isolated from wheat in different countries worldwide, mainly Italy. The strains were phylogenetically identified mostly as belonging to both *Alternaria* and *Infectoriae* sections, in a percentage of approximately 50% for both groups. A particular concern is caused by the species of the *Alternaria* section since most of them produce mycotoxins. In particular, *A. alternata* is the most important mycotoxin producing genus, since these mentioned species are widely reported to produce TA, AME, AOH, ALT and ATX (Logrieco et al., 2009; Patriarca et al., 2019). Therefore, *A. alternata* accumulation in wheat kernels is a serious risk for human consumption. Furthermore, our data demonstrated a lack of correlation between *Alternaria* species occurrence and geographical origin, showing that the risk is similar at worldwide level, maybe also for the ability of this species to grow under a wide range of environmental conditions.

The present study also reports interesting data related to *Alternaria* taxonomy. For decades, morphological traits (such as conidial shape and conidia morphology), under given environmental and culture conditions, have been the only suitable tools to characterize and identify more than 280 *Alternaria* species (Simmons, 1967, 1992, 2007). However, the high sensitivity of *Alternaria* to environmental factors may lead to ambiguities if taxonomy is based only on the evaluation of these phenotypic traits (Andrew et al., 2009). In particular, the morphological identification of *Alternaria* is often problematic, due to several interspecific similarities and intraspecific polymorphism of the characters considered for establishing species boundaries (Andersen et al., 2015; Hong et al., 2006; Poursafar et al., 2018; Pryor and Michailides, 2002; Roberts et al., 2000; Simmons, 1990b; Simmons and Roberts, 1993). The use of molecular techniques in taxonomy studies helped to have a more reliable classification system even though, in the case of *Alternaria*, they were not as robust as for other fungal genera such as *Fusarium* (O'Donnell et al., 2015), *Aspergillus* (Samson et al., 2007), and *Penicillium* (Visagie et al., 2014). Indeed, molecular tools helped to discriminate with a good efficiency *Alternaria* genus from closely related genera such as *Stemphylium*, *Nimbya* and *Embellisia* (Lawrence et al., 2012; Woudenberg et al., 2013), and to differentiate species sections within the genus, but they were not resolved at a species level (Andrew et al., 2009; Lawrence et al., 2013; Woudenberg et al., 2015).

In our study, we characterized, through multilocus sequence analyses, *Alternaria* strains isolated from wheat kernels. However, the lack of availability of *Alternaria* appropriate reference sequences to be used for a robust comparison with unidentified strains, make it very difficult to assign a species identity. This is also due to the fact that to date, a pool of genes unanimously used or more suitable for phylogenetic studies on *Alternaria* has not been defined yet. In several reports, different authors have used different genes or excluded some of them from the analyses due to the inconsistent results obtained. As an example, the housekeeping genes *tef*, *tub* and *RPB2* were excluded by Lawrence et al. (2013), but included by Woudenberg et al. (2015), to build a robust molecular phylogeny within the *Alternaria* section of the genus. To compare the *Alternaria* unknown strain with all the known *Alternaria* species, we amplified and sequenced some reference strains of species occurring on wheat available in our ITEM Collection or received by other Fungal Collections (*A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria* and *A. triticina*). On the other hand we compared our data with the genomes of 20 further *Alternaria* species from the AGD (Table 3; Dang et al., 2015). However, we could consider only species for which the genome was sequenced, that mainly belonged to *Alternaria* and *Porri* sections. Also, confusing data do exist in sequence databases for erroneous assignment of species names due to the previous morphological misidentifications. As an example, in our study, the strain MUCL 44259, received as *A. triticina* from the MUCL Collection, was reported as *A. arborescens* by Mercado Vergnes et al. (2006). Indeed, MUCL 44259 in our data resulted closely related to *A. citriarbusti*, *A. mali*, *A. gaisen*, *A. fragariae*, *A. longipes* and *A. tangelonis* reference strains in our phylogenetic tree (Fig. 2). This incongruity underlines that it is still a crucial issue to provide reference material genetically well defined for investigations in *Alternaria* genus, that should be accurately validated by a standardized common approach by scientists dealing with *Alternaria* taxonomy.

Our data confirmed the clear differentiation between *Alternaria* and *Infectoriae* sections (Fig. 1).

Moreover, the phylogenetic tree focused on *Alternaria* section (Fig. 2) clearly shows that the previously described species *A. alternata*, *A. tenuissima*, *A. turkisafria* and *A. limoniasperae* share same sequences. Since the above mentioned species were reported indistinguishable by Woudenberg et al. (2015), that compared their whole genomes and transcriptomes, we agree with the proposal to merge these species in the same species, namely *A. alternata*. Furthermore, *A. arborescens*, defined by Woudenberg et al. (2015) as a species complex, formed a distinct clade from *A. alternata* also in our analyses. On the other hand, while *A. citriarbusti* and *A. mali*, were proposed by Woudenberg et al. (2015) as synonymous of *A. alternata* species, in our study these two species were grouped with *A. gaisen*, *A. fragariae*, *A. longipes* and *A. tangelonis* (syn. *A. gossypina*, Woudenberg et al., 2015), that were well defined as different species, distinguishable from *A. alternata* (Fig. 2). The incongruence of our results compared to data generated by Woudenberg et al. (2015) could be likely due to the different genomic regions considered for phylogenetic analysis. Thus, in our opinion, until a set of common genes and procedures are not unanimously and well defined by scientific community dealing with *Alternaria* genus taxonomy the phylogenetic relationships among species will remain confused.

The phylogenetic tree focused on *Infectoriae* section (Fig. 3), that includes *A. infectoria* and *A. triticina* species, confirmed a higher genetic variability of this section if compared to *Alternaria* section. Interestingly, only among the strains from Italy analyzed in our work (Table 1), the section *Infectoriae* strains, prevailed *Alternaria* section strains, therefore showing a lower contamination of wheat by mycotoxin producing species in Italy. Also, it is important to underline a high presence of *A. triticina* species on wheat kernels sampled in Italy, since this species has been reported to occur only on leaves (Perello and Larran, 2013).

The phylogenetic studies on *Infectoriae* section proved that this section consists of a wide variety of taxa, morphologically and phylogenetically differentiated (Andersen et al., 2009; Woudenberg et al., 2013). Recently, Lawrence et al. (2014) deeply investigated *A. infectoria* species group, resolving it in 25 species, that clustered in a well-defined clade phylogenetically very distant from the clade composed by the other *Alternaria* species-groups. Our results also show the very high variability within the *Infectoriae* section and its clear phylogenetic distance from other sections of the genus *Alternaria*.

Infectoriae section is characterized also by the fact that includes species all known to produce a sexual state (previously assigned to *Lewia*, Simmons, 1989, 2007), on the contrary of the other *Alternaria* sections that do not have sexual stage. Moreover, *Infectoriae* section was demonstrated to be more phylogenetically more closely related to *Pseudoalternaria*, *Chalatospora*, *Embellisia* (group I and II) and *Nimbya* genera than to *Alternaria* section (Lawrence et al., 2012, 2013, 2014; Woudenberg et al., 2013). Furthermore, species belonging to *Infectoriae* section are proved that are not able of producing mycotoxins, being known for producing secondary metabolites such as novae-zelandins and infectopyrone, unique to this section species and never reported for species belonging to the other *Alternaria* section species (Christensen et al., 2005).

Therefore, taking in consideration the typical morphology and cultural characteristics, the chemical characterization, the sexuality, and the several studies demonstrating the clear phylogenetic separation and the paraphyly of *Infectoriae* section towards the other *Alternaria* sections, we consider worthwhile to sequence further genes with a perspective to evaluate the possibility to place *Infectoriae* section in a

different genus than *Alternaria* or to consider it as genus apart.

5. Conclusions

Morphological traits alone are too much variable for *Alternaria* species identification. However, this tool was almost the unique approach for the *Alternaria* and closely related genera for species identification, causing taxonomic confusion and misidentification even when gene sequences were used for confirming eventual reference strains previously morphologically identified wrongly. Therefore, we strongly recommend a multilocus sequence approach for a wide re-classification and taxonomy re-definition not only in *Alternaria* genus but also in the whole *Pleosporaceae* family.

Isolates were grouped in two main clades: the *Alternaria* section, including *A. alternata*, *A. tenuissima* and *A. arborescens* species, and the *Infectoriae* section, that includes the two species *A. infectoria* and *A. triticina*.

The *Alternaria* species resulted not geographically specific, being all spread at worldwide level.

Furthermore, there is a major need to consider the eventuality that species showing coincident gene sequences and phylogenetically indistinguishable would be consider same genetic entities. This approach should allow to reduce the number of defined *Alternaria* species, which division is often based only on the morphological traits. Finally, we believe that there is a need of an international agreement among taxonomists dealing with *Alternaria* species on the possible re-classification of section *Infectoriae*, since the genetic and the mycotoxin profile studies that were generated by several research groups, including our report, would suggest to place this section in another genus than *Alternaria*.

References

Amatulli, M.T., Fanelli, F., Moretti, A., Mulè, G., Logrieco, A., 2013. *Alternaria* species and mycotoxins associated to black point of cereals. Mycotoxins 63 (1), 39–46.

Andersen, B., Sørensen, J.L., Nielsen, K.F., Gerrits van den Ende, B., de Hoog, S., 2009. A polyphasic approach to the taxonomy of the *Alternaria infectoria* species-group. Fungal Genet. Biol. 46 (9), 642–656.

Andersen, B., Nielsen, K.F., Fernandez Pinto, V., Patriarca, A., 2015. Characterization of *Alternaria* strains from Argentinean blueberry, tomato, walnut and wheat. Int. J. Food Microbiol. 196, 1–10.

Andrew, M., Peever, T.L., Pryor, B.M., 2009. An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. Mycologia 101 (1), 95–109.

Asam, S., Habler, K., Rychlik, M., 2013. Determination of tenuazonic acid in human urine by means of a stable isotope dilution assay. Anal. Bioanal. Chem. 405 (12), 4149–4158.

Aust, H.J., Bashi, E., Rotem, J., 1980. Flexibility of plant pathogens in exploiting ecological and biotic conditions in the development of epidemics. In: Palti, J., Kranz, J. (Eds.), Comparative Epidemiology. Pudoc, Wageningen, pp. 46–56.

Bensassi, F., Zid, M., Rhouma, A., Bacha, H., Hajlaoui, M.R., 2009. First report of *Alternaria* species associated with black point of wheat in Tunisia. Ann. Microbiol. 59, 465–467.

Berbee, M.L., Pirseyedi, M., Hubbard, S., 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia 91, 964–977.

Christensen, K.B., Van Klink, J.W., Weavers, R.T., Larsen, T.O., Andersen, B., Phipps, R.K., 2005. Novel chemotaxonomic markers of the *Alternaria infectoria* species-group. J. Agric. Food Chem. 53 (24), 9431–9435.

CPC, 2018. *Alternaria triticina* Datasheet. Crop Protection Compendiumhttps://www.cabi.org/cpc/.

da Cruz Cabral, L., Fernandez Pinto, V., Patriarca, A., 2016. Control of infection of tomato fruits by *Alternaria* and mycotoxin production using plant extracts. Eur. J. Plant Pathol. 145 (2), 363–373.

Dang, H.X., Pryor, B., Peever, T., Lawrence, C.B., 2015. The *Alternaria* genomes database: a comprehensive resource for a fungal genus comprised of saprophytes, plant pathogens, and allergenic species. BMC Genomics 16 (1), 239.

Davis, V.M., Stack, M.E, 1991. Mutagenicity of *stemphyltoxin III*, a metabolite of Alternaria alternata. Appl. Environ. Microbiol. 57, 180–182.

EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011. Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. EFSA J. 9, 2407.

Fleck, S.C., Sauter, F., Pfeiffer, E., Metzler, M., Hartwig, A., Köberle, B., 2016. DNA damage and repair kinetics of the *Alternaria* mycotoxins alternariol, altertoxin II and stemphyltoxin III in cultured cells. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 798–799, 27–34.

Gannibal, P.B., Klemsdal, S.S., Levitin, M.M., 2007. AFLP analysis of Russian *Alternaria tenuissima* populations from wheat kernels and other hosts. Eur. J. Plant Pathol. 119, 175–182.

Gargouri-Kammoun, L., Bensassi, F., Mnari-Hattab, M., Rhouma, A., Bacha, H., Hajlaoui, M.R., 2014. Identification of *Alternaria* species recovered from stored durum wheat kernels in Tunisia. Tunis. J. Plant Prot. 9, 119–129.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Guo, Y.L., 2005. Anamorphic fungi. In: Zhuang, W.Y. (Ed.), Fungi of Northwestern China. Mycotaxon Ltd., Ithaca NY, pp. 125–232.

Hong, S.G., Cramer, R.A., Lawrence, C.B., Pryor, B.M., 2005. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. Fungal Genet. Biol. 42, 119–129.

Hong, S.G., Maccaroni, M., Figuli, P.J., Pryor, B.M., Belisario, A., 2006. Polyphasic classification of *Alternaria* isolated from hazelnut and walnut fruit in Europe. Mycol. Res. 110, 1290–1300.

Huybrechts, I., De Ruyck, K., De Saeger, S., De Boevre, M., 2018. Uniting large-scale databeses to unravel the impact of chronic multi-mycotoxins exposures on colorectal cancer incidence in Europe. In: Proceedings of the 2nd MycoKey International Conference, Wuhan, China, 16–18 September 2018. China Agricultural Science and Technology Press, Beijing, China, pp. 181–183.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874.

Lawrence, D.P., Park, M.S., Pryor, B.M., 2012. *Nimbya* and *Embellisia* revisited, with nov. comb for *Alternaria celosiae* and *A. perpunctulata*. Mycol. Prog. 11, 799–815.

Lawrence, D.P., Gannibal, P.B., Peever, T.L., Pryor, B.M., 2013. The sections of *Alternaria*: formalizing species-group concepts. Mycologia 105, 530–546.

Lawrence, D.P., Gannibal, P.B., Dugan, F.M., Pryor, B.M., 2014. Characterization of *Alternaria* isolates from the infectoria species-group and a new taxon from *Arrhenatherum*, *Pseudoalternaria arrhenatheria* sp. nov. Mycol. Prog. 13 (2), 257–276.

Lehmann, L., Wagner, J., Metzler, M., 2006. Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. Food Chem. Toxicol. 44, 398–408.

Li, F., Yoshizawa, T., 2000. *Alternaria* mycotoxins in weathered wheat from China. J. Agric. Food Chem. 48, 2920–2924.

Liu, G.T., Qian, Y.Z., Zhang, P., Dong, Z.M., Shi, Z.Y., Zhen, Y.Z., Miao, J., Xu, Y.M., 1991. Relationships between *Alternaria alternata* and oesophageal cancer. IARC Sci. Publ. 105, 258–262.

Logrieco, A., Moretti, A., Solfrizzo, M., 2009. *Alternaria* toxins and plant diseases: an overview of origin and occurrence. World Mycotoxin J. 2, 129–140.

Mašková, Z., Tančinová, D., Barboráková, Z., Felšöciová, S., Císarová, M., 2012. Comparison of occurrence and toxinogenity of *Alternaria* spp. isolated from samples of conventional and new crossbred wheat of Slovak origin. J. Microbiol. Biotechnol. Food Sci. 1, 552–562.

Mercado Vergnes, D., Renard, M.E., Duveiller, E., Maraite, H., 2006. Identification of *Alternaria* spp. on wheat by pathogenicity assays and sequencing. Plant Pathol. 55, 485–493.

Misaghi, I.J., Grogan, R.G., Duniway, J.M., Kimble, K.A., 1978. Influence of environment and culture media on spore morphology of *Alternaria alternata*. Phytopathology 68, 29–34.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7, 103–116.

O'Donnell, K., Ward, T.J., Robert, V.A.R.G., Crous, P.W., Geiser, D.M., Kang, S., 2015. DNA sequence-based identification of *Fusarium*: current status and future directions. Phytoparasitica 43 (5), 583–595.

Ostry, V., 2008. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. World Mycotoxin J. 1, 175–188.

Park, D.S., Sayler, R.J., Hong, Y.G., Nam, M.H., Yang, Y., 2008. A method for inoculation and evaluation of rice sheath blight disease. Plant Dis. 92, 25–29.

Patriarca, A., Azcarate, M.P., Terminiello, L., Pinto, V.F., 2007. Mycotoxin production by *Alternaria* strains isolated from Argentinean wheat. Int. J. Food Microbiol. 119, 219–222.

Patriarca, A., da Cruz Cabral, L., Pavicich, M.A., Nielsen, K.F., Andersen, B., 2019. Secondary metabolite profiles of small-spored *Alternaria* support the new phylogenetic organization of the genus. Int. J. Food Microbiol. 2019, 135–143.

Peever, T.L., Su, G., Carpenter-Boggs, L., Timmer, L.W., 2004. Molecular systematic of citrus-associated *Alternaria* species. Mycologia 96, 119–134.

Perello, A., Larran, 2013. Nature and effect of alternaria spp. complex from wheat grain on germination and disease transmission. Pak. J. Bot. 45 (5), 1817–1824.

Poursafar, A., Ghosta, Y., Orina, A.S., Philipp, B.G., Javan-Nikkhah, M., Lawrence, D.P., 2018. Taxonomic study on *Alternaria* sections *Infectoriae* and *Pseudoalternaria* associated with black (sooty) head mold of wheat and barley in Iran. Mycol. Prog. 17 (3), 343–356.

Prasada, R., Prabhu, A., 1962. Leaf blight of wheat caused by a new species of *Alternaria*. Indian Phytopathol. 15, 292–293.

Pryor, B.M., Michailides, T.J., 2002. Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. Phytopathology 92, 406–416.

Rathod, S.R., Chavan, A.M., 2010. Incidence of *Alternaria* species on different cereals, pulses and oil seeds. J. Ecobiotechnol. 2 (6), 63–65.

Roberts, R.G., Reymond, S.T., Andersen, B., 2000. RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species groups.

Mycol. Res. 104, 151–160.
Rotem, J., Cohen, Y., Bashi, E., 1978. Host and environmental influences on sporulation in vivo. Annu. Rev. Phytopathol. 16, 3–101.
Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black aspergilla. Stud. Mycol. 59, 129–145.
Schrader, T.J., Cherry, W., Soper, K., Langlois, I., Vijay, H.M., 2001. Examination of *Alternaria alternata* mutagenicity and effects of nitrosylation using the Ames Salmonella test. Teratog. Carcinog. Mutagen. 21, 261–274.
Schrader, T.J., Cherry, W., Soper, K., Langlois, I., 2006. Further examination of the effects of nitrosylation on *Alternaria alternata* mycotoxin mutagenicity in vitro. Mutat. Res. 606, 61–71.
Scott, P.M., Zhao, W., Feng, S., Lau, B.P.-Y., 2012. *Alternaria* toxins alternariol and alternariol monomethyl-ether in grain foods in Canada. Mycotoxin Res. 28, 261–266.
Sharma, A.K., Singh, D.P., Kumar, J., Singh, A.K., Nagarajan, S., 1998. Progress Report 1997–98. Vol. V Plant Protection (Pathology and Nematology). Directorate of Wheat Research, Karnal, India.
Shukla, P.K., Pandey, M.K., Pandey, P.S., Singh, C.M., 2006. Occurrence of leaf blight of wheat in reclaimed usar soils. Indian Phytopathol. 59 (2), 240–242.
Simmons, E.G., 1967. Typification of *Alternaria*, *Stemphylium*, and *Ulocladium*. Mycologia 59, 67–92.
Simmons, E.G., 1989. Macrospora Fuckel (*Pleosporales*) and related anamorphs. Sydowia 41, 314–329.
Simmons, E.G., 1990a. *Embellisia* and related teleomorphs. Mycotaxon 38, 251–265.
Simmons, E.G., 1990b. *Alternaria* themes and variations (17–53). Mycotaxon 37, 79–119.
Simmons, E.G., 1992. *Alternaria* taxonomy: current status, viewpoint, challenge. In: Chelkowski, J., Visconti, A. (Eds.), *Alternaria*: Biology, Plant Diseases and Metabolites. Elsevier Science Publishers, New York, pp. 1–36.
Simmons, E.G., 1999. *Alternaria* themes and variations (236–243): host specific toxin producers. Mycotaxon 70, 325–369.
Simmons, E.G., 2007. *Alternaria*: An Identification Manual. CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.
Simmons, E.G., Roberts, R., 1993. *Alternaria* themes and variations (73). Mycotaxon 48, 109–140.
Solhaug, A., Wisbech, C., Christoffersen, T.E., Hult, L.O., Lea, T., Eriksen, G.S., Holme, J.A., 2015. The mycotoxin alternariol induces DNA damage and modify macrophage phenotype and inflammatory responses. Toxicol. Lett. 239, 9–21.
Somma, S., Pose, G., Pardo, A., Mulè, G., Fernandez Pinto, V., Moretti, A., Logrieco, A.F., 2011. AFLP variability, toxin production, and pathogenicity of *Alternaria* species from Argentinean tomato fruits and puree. Int. J. Food Microbiol. 145, 414–419.
Thomma, B.P.H.J., 2003. Alternaria spp.: from general saprophyte to specific parasite. Mol. Plant Pathol. 4 (4), 225–236.
Tralamazza, S.M., Piacentini, K.C., Iwase, C.H.T., Rocha, L.D.O., 2018. Toxigenic *Alternaria* species: impact in cereals worldwide. Curr. Opin. Food Sci. 23, 57–63.
Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., Samson, R.A., 2014. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78, 343–371.
Vučković, J.N., Brkljača, J.S., Bodroža-Solarov, M.I., Bagi, F.F., Stojšin, V.B., Ćulafić, J.N., Aćimović, M.G., 2012. *Alternaria* spp. on small grains. Food Feed Res. 39, 79–88.
Wang, W., Jones, C., Ciacci-Zanella, J., Holt, T., Gilchrist, D.G., Dickman, M.B., 1996. Fumonisins and *Alternaria alternata lycopersici* toxins: Sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. Proc. Natl. Acad. Sci. USA 93, 3461–3465.
Wiese, M.V., 1987. Compendium of Wheat Diseases, second ed. APS Press, St. Paul., Minn.
Woudenberg, J.H.C., Groenewald, J.Z., Binder, M., Crous, P.W., 2013. *Alternaria* redefined. Stud. Mycol. 75, 171–212.
Woudenberg, J.H.C., Seidl, M.F., Groenewald, J.Z., de Vries, M., Stielow, J.B., Thomma, B.P.H.J., Crous, P.W., 2015. *Alternaria* section *Alternaria*: species, formae speciales, or pathotypes? Stud. Mycol. 82, 1–21.
Yekeler, H., Bitmis, K., Ozcelik, N., Doymaz, M.Z., Calta, M., 2001. Analysis of toxic effects of *Alternaria* toxins on oesophagus of mice by light and electron microscopy. Toxicol. Pathol. 29, 492–497.
Zhou, B., Qiang, S., 2008. Environmental, genetic and cellular toxicity of tenuazonic acid isolated from *Alternaria alternata*. Afr. J. Biotechnol. 7, 1151–1156.